RESEARCH ARTICLE

Evolution of Antidrug Antibody Assays During the Development of Anti-Tissue Factor Pathway Inhibitor Monoclonal Antibody Marstacimab

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Abstract

Tissue factor pathway inhibitor (TFPI) is an endogenous inhibitor of the extrinsic coagulation pathway. In patients with hemophilia A or B, inhibition of TFPI is an alternative therapeutic approach that augments the extrinsic coagulation pathway. Marstacimab is an investigational fully human monoclonal antibody that binds and neutralizes TFPI and is being evaluated as a prophylactic treatment to prevent or reduce the frequency of bleeding episodes in patients with severe hemophilia A or B, with or without inhibitors (antibodies against coagulation factors). However, the efficacy, safety, and pharmacokinetics of marstacimab may be affected by the induction of antidrug antibody (ADA) responses. Here, we describe the evolution and validation of three quasi-quantitative electrochemiluminescence-based methods to detect marstacimab ADAs, starting from their use in a first-in-human phase 1 study to their use in phase 2 and 3 clinical studies of patients with severe hemophilia. For all three methods, validation criteria evaluated the performance of the assays in screening and confirmatory cut points, precision, selectivity, drug tolerance, target interference, and stability. Additional criteria for validation were dilution linearity (Methods 1 and 2) and low positive control concentration, prozone effect, plate homogeneity, and robustness (Method 3). The three methods met validation criteria and are a potentially valuable tool in detecting the induction of marstacimab ADAs during treatment in patients with hemophilia.

Keywords antibodies · hemophilia A · hemophilia B · neutralizing · pharmacokinetics

Introduction

A risk associated with the use of protein-based drugs is the induction of an antidrug antibody (ADA) response, which may alter pharmacokinetics, neutralize the therapeutic effect of the drug, and/or cause adverse effects (1, 2). ADA assessment is an important aspect of drug development and drug safety evaluation during clinical trials (1, 2). The availability of appropriate methods of ADA detection and characterization is critical for accurate immunogenicity assessments (3).

An alternative approach to coagulation factor VIII (FVIII) or factor IX (FIX) replacement in patients with hemophilia is to target augmentation of the extrinsic coagulation pathway (4–6). Marstacimab (also known as PF-06741086) is an investigational, fully human monoclonal immunoglobulin G1 (IgG1) that targets the Kunitz-2 domain of tissue factor pathway inhibitor (TFPI), a serine protease inhibitor that negatively regulates the extrinsic coagulation pathway (6). Low plasma TFPI concentrations have been associated with decreased clotting times (7). Thus, a reduction in TFPI-mediated inhibition of the extrinsic coagulation pathway by marstacimab is expected to increase clotting activity in patients with bleeding disorders. Marstacimab is being investigated as a prophylactic treatment to prevent or reduce the frequency of bleeding episodes in patients with severe hemophilia A or B with or without inhibitors (neutralizing antibodies to FVIII or FIX) (8). Given that it is a protein-based therapeutic, determining its ADA response potential and any related clinical sequelae of ADA development are necessary.

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Risk assessment of marstacimab for antibody response induction was based on its sequence, physical characteristics, route of administration, and the planned clinical indication (2). Marstacimab is a human antibody derived from a phage display library generated from a healthy volunteer. Three mutations (L234A, L235A, and G237A; EU numbering system) were engineered in the fragment crystallizable (Fc) region to minimize effector function (9, 10). Since these are non-germline mutations, their introduction may increase the risk of ADAs. Marstacimab has no abnormal post-translational modifications. No unusual degradation or aggregation has been found for marstacimab (unpublished data). This agent is designed for subcutaneous (SC) administration. Because marstacimab is a fully human IgG1 with no endogenous counterpart, the ADA response, if developed, is expected to primarily affect the pharmacokinetics and/or efficacy of the compound.

A tiered approach to ADA response testing was initially followed, using screening, confirmation, titer, and analytic protocols continuously evaluated for performance. Samples confirmed positive for ADA were also evaluated for neutralizing antibody (NAb), the assay for which is beyond the scope of this article. Here, we report on three ADA-detecting protocols used for marstacimab. In the first-in-human study of marstacimab (ClinicalTrials.gov identifier: NCT02531815), ADA responses were evaluated in plasma samples from healthy male volunteers who received single escalating doses of the drug (ADA assay Method 1) (11). Assay methodologies for marstacimab ADAs continued to be optimized to mitigate matrix interference in later-stage clinical trials. Here we describe the evolution of the marstacimab ADA assay methodologies and their validation, from the first study in humans (ADA assay Method 1) (11), to the phase 1b/2 study in patients with severe hemophilia

(ClinicalTrials.gov identifier: NCT02974855; ADA assay Method 2) (12), and the phase 3 study in patients with severe hemophilia (ClinicalTrials.gov identifier NCT03938792; ADA assay Method 3).

Materials and Methods

Method 1: First-in-Human Phase 1 Study

Electrochemiluminescent-Based Binding Antibody Assay

The electrochemiluminescence (ECL) assay was developed on the Meso Scale Discovery (MSD; Meso Scale Diagnostics; Gaithersburg, MD, USA) platform to detect antibodies that bind specifically to marstacimab in sodium citrate human plasma samples. The MSD platform-based bridging ECL assay is a quasi-quantitative, ligand-binding assay (13).

All critical reagents used in the ADA assay were generated by Pfizer Inc (Andover, MA, USA). Streptavidin-coated MSD plates were blocked with 4% bovine serum albumin-phosphate buffer saline, and samples or controls were diluted $25\times$ in 300 mM acetic acid and incubated for 30 min to allow dissociation of ADAs and circulating marstacimab. Dissociated samples were then added to dilution plates containing a master mix of biotinylated marstacimab, ruthenylated marstacimab and Tris pH 8.5 in assay buffer at $3\times$ dilution and incubated for 1 h. This enabled marstacimab ADA in the sample to bind to both the biotinylated (0.9 mg/mL) and ruthenylated (2.16 mg/mL) marstacimab to form an antibody complex bridge (Fig. 1). After the MSD plate was washed (wash buffer: 50 mM Tris, 500 mM Sodium Chloride, 1 mM Glycine, $+0.05\%$ (v/v) Tween-20, pH 7.2 ± 0.1), samples were transferred from the dilution plate to the MSD

Fig. 1 Electrochemiluminescence bioanalytical method for the detection of antidrug antibodies. Schematic of assay developed to detect antidrug antibodies in healthy volunteers treated with marstacimab. *ECL*, electrochemiluminescence; *MSD*, Meso Scale Discovery
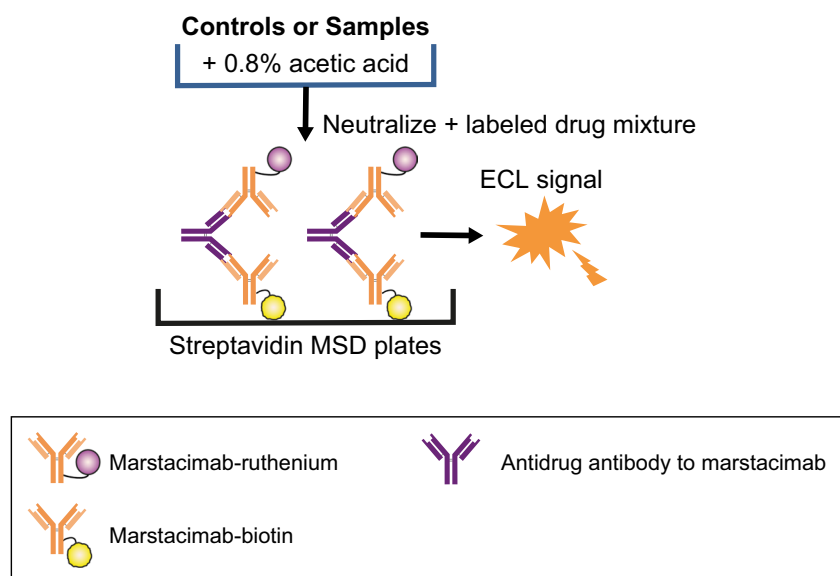
plate and incubated for 1 h. Plates were washed 4 times followed by the addition of the MSD Gold Read Buffer. In the presence of tripropylamine (TPA)-containing Read Buffer, ruthenium produces a chemiluminescent signal when voltage is applied. Chemiluminescence was measured in relative luminescence units (RLU) on an MSD Sector Imager 6000 microplate reader.

Samples were considered positive if the mean RLU was greater than or equal to the calculated screening cut point. Samples testing positive were run in a confirmatory assay in which excess marstacimab unlabeled drug (5.11 μg/mL) was added in a competitive binding format to demonstrate binding specificity, defined as percent inhibition greater than or equal to the confirmation cut point. To be considered positive for ADA, the sample must have tested positive in both screening and confirmatory steps as well as in a titer assay.

A sample analysis run included negative controls (NCs) at $n = 8$ (single reading); primary positive controls (PCs) at $n = 1$ (duplicate readings), 1:75 minimum required dilution (MRD) after addition of master mix, and seven additional PC serial dilutions at 1:3; and for the marstacimab confirmation test only, marstacimab ADA (high PC [HPC] and low PC [LPC]) incubated in the presence or absence of marstacimab spiked into the master mix ($n \geq 1$).

ADA Assay Performance Validation

Assay performance was characterized using rabbit anti-marstacimab antiserum as the PC and pooled normal sodium citrate human plasma as the NC. Assay validation included assessment of assay cut points, precision, specificity, sensitivity, matrix interference, drug tolerance, and stability. Cut points were determined using 50 commercially available normal sodium citrate human plasma samples not exposed to marstacimab that were diluted 1:75. A floating cut point factor was determined statistically based on the 95% upper confidence limit after removal of statistical outliers. Outliers were identified using the box-plot approach in JMP Statistical Discovery software (version 10.0, SAS Institute, Inc.; Cary, NC, USA).

Assay precision was expressed as the coefficient of variation (%CV) from analyzing replicates of PCs and NCs. The assay was considered precise if the %CV of the PC end point $\log_{10}$ titers from each set of PCs (intra-run) and all accepted runs (inter-run) were less than 25.0%. Intra-run precision was determined from one set of NCs and five sets of independently titrated PCs on each plate. The precision of the end point $\log_{10}$ titer values for the PC was determined from all 5 titration samples tested on the same plate. Intra-day precision was determined from one set of NCs and five sets of PCs on four separate plates. The PC analyzed on each plate was an independent PC titration using the same stock for each plate and sample preparation per plate. The precision

of the end point $\log_{10}$ titer values for the PC was determined from all PC samples analyzed across the four plates. Inter-run precision for the PC was determined from all accepted analytical runs, which were carried out by three analysts.

Specificity was determined as part of the confirmation assay described above. Sensitivity was defined as the concentration of the assay PC in sodium citrate human plasma resulting in a signal equal to the plate cut point and was derived from multiple independent titrations (Table I). Reported sensitivity values were MRD-corrected. Selectivity was performed by analyzing the NC and 10 individual lots of sodium citrate human plasma samples spiked with and without HPC (1:150 dilution) and LPC (1:3750 dilution). Although HPC and LPC were prepared from unpurified polyclonal antiserum, purified anti-marstacimab at a concentration of 500 ng/mL was used for drug and target tolerance testing, as this was a level that was consistently positive in the absence of interferents. Drug tolerance was determined using anti-marstacimab at HPC (500 ng/mL) and marstacimab at eight concentrations between 0 and 200 μg/mL to identify the highest concentration of drug at which the PC still appeared positive or greater than the plate cut point. Target interference testing assessed the concentration of TFPI that inhibits the ability to detect PCs or causes false positive results in absence of PC. The NC and PC (500 ng/mL) were pre-incubated with TFPI (0, 50, 100, and 150 ng/mL), and the highest concentration of TFPI for which the NC appeared negative and PC appeared positive or greater than the plate cut point was identified. This test was repeated with two lots of TFPI at 0, 150, 250, 500, and 1000 ng/mL.

Stability of the PC in sodium citrate human plasma was assessed after 24 h at room temperature and after five cycles of freezing ($-20°C$ and $-70°C$, ≥ 24 h for the first cycle and ≥ 12 h for subsequent cycles) and thawing (unassisted at room temperature). Samples were analyzed together with an aliquot of the PC that was thawed immediately before analysis.

Method 2: Phase 2 Study

Electrochemiluminescent-Based Binding Antibody Assay with Affinity Capture Elution and Tissue Factor Protease Inhibitor Blocking

Samples containing anti-marstacimab antibody (PC antibody), NC plasma, and study specimens were diluted 1:5 using 1% bovine serum albumin/phosphate buffered saline/Tween pH 7.4 (BSA/PBST;) and then 40 μL of the diluted sample was added to 150 μL of 100 mM glycine pH 2.0 (Fig. 2). Acidified samples were incubated with 10 μL marstacimab–biotin (final concentration 5 μg/mL), then 80 μL of this mixture was introduced to a streptavidin-coated high binding capacity plate containing 8.5 μL of 1M Tris

Table I Key Highlights of Antidrug Antibody Assay Validation for Method 1 (Electrochemiluminescence Antidrug Antibody Assay Validation), Method 2 (Electrochemiluminescence-Based Binding Assay with Affinity Capture Elution and Tissue Factor Protease Inhibitor Blocking), and Method 3 (Electrochemiluminescence-Based Binding Assay With Affinity Capture Elution and Modified Blocking)

Characteristic	Method 1	Method 2	Method 3
MRD	1:75	1:34	1:34
PC	Rabbit polyclonal (unpurified antiserum)	Mouse monoclonal	Mouse monoclonal
LPC	1:3750	180 ng/mL	110 ng/mL
HPC	1:150	1620 ng/mL	1620 ng/mL
Sample pretreatment	Acid dissociation in acetic acid, pH 3.0	Acid dissociation in glycine, pH 2.0, followed by ACE	Acid dissociation in acetic acid, pH 3.0, followed by ACE
Blocking agents	None	Mouse anti-TFPI	Mouse anti-TFPI and Chromepure hIgG
Screening cut point	1.19	1.24	1.26
Confirmatory cut point	13.3%	70.4%	25.6%
Sensitivity[a]	18.3 ng/mL	245 ng/mL[b]	110 ng/mL
Inter-run precision (%CV)			
Screening	4.6% for HPC (endpoint titer)	4.0% for HPC (endpoint titer)	6.5% for LPC (S/N) 16.5% for HPC (S/N)
Confirmatory	28.7% for LPC 0.3% for HPC	2.6% for LPC 2.5% for HPC	5.5% for LPC 2.5% for HPC
Selectivity (# of samples passing acceptance criteria)	10/10 normal 10/10 disease state	10/10 disease state	10/10 disease state 6/6 normal hemolyzed 6/6 normal lipemic
Interference			
Drug tolerance (No false negative)	100 µg/mL drug at 500 ng/mL PC	200 µg/mL at 400 ng/mL PC	100 µg/mL at 110 ng/mL PC 300 µg/mL at 250 ng/mL PC 400 µg/mL at 1620 ng/mL PC
Target interference (No false positive)	< 50 ng/mL in NC 250 ng/mL in NC (using purified TFPI)	1200 ng/mL in NC	2000 ng/mL in NC
Impact of method change	Original method	Reduce false positives. ADA incidence reduced from 47% to 0–11.5%	Confirmatory cutpoint reduced from 70.4% to 25.6%. Effect on ADA incidence TBD

[a]Sensitivities reported were from different PC

[b]182 ng/mL without statistical outliers

ACE, antigen capture elution; *ADA*, antidrug antibody; *CV*, coefficient of variation; *HPC*, high positive control; *LPC*, low positive control; *MRD*, minimum required dilution; *NC*, negative control; *PC*, positive control; *S/N*, signal-to-noise; *TFPI*, tissue factor pathway inhibitor

pH 8.5 neutralization buffer, causing marstacimab ADAs to be retained on the plate. After a wash step, marstacimab ADAs were eluted with 85 µL of 100 mM glycine pH 2.0. The eluent (70 µL) was mixed and incubated with 20 µL of Master Mix (2.25 µg/mL ruthenium-labeled marstacimab (marstacimab-RU), 2.25 µg/mL biotin-labeled marstacimab (marstacimab-biotin), and 64.5 µg/mL mouse anti-TFPI IgG1 [Target Blocker 7A4]) in 1% BSA and 1M Tris pH 8.5 buffer. During this incubation, TFPI from samples would bind 7A4 while the marstacimab-RU and marstacimab-biotin would bind to marstacimab ADAs. Samples were then added to blocked and washed MSD streptavidin plates. Marstacimab ADAs were detected using tripropylamine-containing read buffer and MSD equipment. The resulting chemiluminescence was measured in RLUs that were proportional to the amount of ADAs present in the plasma samples.

ADA Assay Performance Validation

Assay performance was characterized using mouse anti-marstacimab antibody clone 106–04 as the PC and pooled normal sodium citrate human plasma as the NC. Assay validation included evaluation of precision, assay cut points, PC stability, matrix selectivity, drug tolerance, target interference, dilution linearity, and sensitivity.

Assay precision was expressed as the %CV from analyzing replicates of PCs and NCs. The assay was considered precise if the %CV of PC end point $\log_{10}$ titers from each set of PCs and all accepted runs were less than 25.0%. Intra-run

Fig. 2 Modified electrochemiluminescence bioanalytical method for the detection of antidrug antibodies with affinity capture elution and tissue factor pathway inhibitor blocking step. [1] Acid dissociation and incubation of ADA with marstacimab-biotin; [2] neutralization of ADA-biotin-marstacimab-target on streptavidin high binding capacity plate; [3] acid elution of ADA; [4] neutralization of ADA and incubation with marstacimab-biotin, marstacimab-ruthenium, and target blocker (mouse anti-TFPI IgG [7A4.D9]; ChromPure human IgG added to reduce nonspecific binding in Method 3); [5] ADA complexed with marstacimab-biotin and marstacimab-ruthenium incubation on streptavidin-coated MSD plate. *ADA*, antidrug antibody; *ECL*, electrochemiluminescence; *MSD*, Meso Scale Discovery
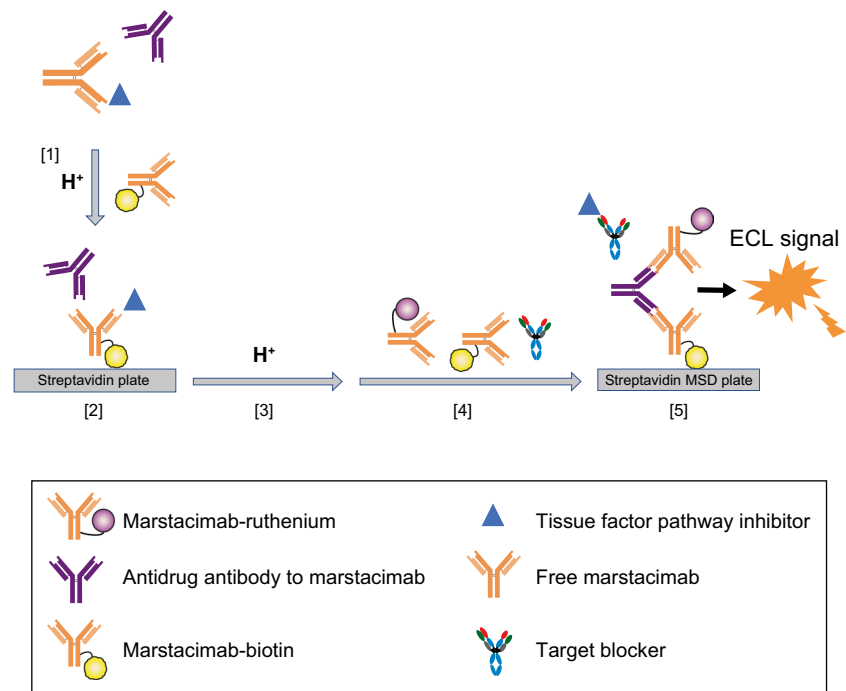
precision was determined from one set of NCs and five sets of independently titrated anti-marstacimab PCs determined from all five titration samples tested on each intra-run plate. The precision of the end point $\log_{10}$ titer values for the PC was determined from all five titration samples on the same plate. Intra-day precision was determined from one set of NCs and five sets of PCs on four separate plates. The PC on each plate was an independent PC titration using the same control stock for each plate and the same preparation per plate. The precision of the end point $\log_{10}$ titer value for PC was determined for all PC samples analyzed across the four plates. Inter-run assay precision for the PC was determined from all 39 accepted analytical runs, which were run by four analysts.

Assay response was evaluated for 30 commercially available sodium citrate human plasma samples not exposed to marstacimab. Samples were subjected to a net total dilution of 1:34, following the assay procedure above, and were assayed in three independent runs among two analysts over at least 3 days. The cut point value was determined based on the 95th percentile of the overall signal-to-noise (S/N) ratio of the 90 individual data points, and was confirmed using 30 sodium citrate human plasma samples incubated in the absence and presence of marstacimab then diluted 1:34 for analysis.

Stability of the anti-marstacimab PC was assessed after 24 h at room temperature and after five cycles of freezing ($-20°C$ and $-70°C$) for at least 24 h in the first cycle and at least 12 h in other cycles and subsequent unassisted

thawing at room temperature. Samples were analyzed after fifth cycle completion together with a PC aliquot that was prepared fresh before analysis. Matrix selectivity was performed by analyzing NCs and 10 individual lots of sodium citrate plasma samples from hemophilia patients spiked with and without LPC (180 ng/mL) and HPC (1620 ng/mL) anti-marstacimab antibodies. Drug tolerance testing was performed using marstacimab at 0, 3.13, 6.25, 12.5, 25, 50, 100, and 200 µg/mL. The highest concentration of marstacimab in which the PC still appeared positive or greater than the plate cut point for each PC was identified. Target interference was determined using anti-marstacimab at NC, LPC, and HPC concentrations and TFPI at 0, 125, 250, 1000 and 1200 ng/mL to identify the highest concentration of TFPI in which the PC still appears positive or greater than the plate cut point. The LPC concentration was set to a level that fell below the cut point ~1% of the time as specified by FDA guidance that was not published at the time of Method 1 (14).

Dilution linearity was assessed to ensure that the PC could be diluted with human plasma without affecting the final calculated concentration. PCs were prepared at 8.1, 2.7, 0.9, and 0.3 µg/mL and diluted past cut point. The end point titers of the PC samples were calculated and compared. Sensitivity was defined as the concentration of anti-marstacimab antibodies in sodium citrate human plasma that result in a signal equal to the plate cut point. Sensitivity was reported from 12 independent titrations and was calculated as the mean PC concentration at plate cut point plus 1.645 ● SD.

Method 3: Phase 3 Study

Electrochemiluminescent-Based Binding Antibody Assay with Affinity Capture Elution and Tissue Factor Protease Inhibitor Blocking

Method 3 was similar to Method 2, except that Method 3 used ChromPure human IgG (Jackson ImmunoResearch, 100 ug/mL) as an additional specific blocking reagent (Fig. 2).

ADA Assay Performance Validation

As with Method 2, assay performance was characterized using mouse anti-TFPI antibody clone 106–04 as the PC and pooled normal sodium citrate human plasma as the NC. Validation assessments included evaluation of precision, assay cut points, PC stability, selectivity (matrix, hemolytic, and lipemic recovery), specificity (drug tolerance and target interference), sensitivity, LPC calculation, prozone effect, plate homogeneity, and robustness.

Inter-run precision was determined from acceptable validation runs of the NC, LPC (110 ng/mL), and HPC (1620 ng/mL). The acceptance criterion for inter-run precision was up to 20% CV for the LPC and HPC (S/N and percent inhibition) and NC. Intra-run precision was analyzed in the screening and confirmatory assay formats for the NC, LPC, and HPC.

The screening cut point differentiated a putative positive sample (defined as having a screening [S/N] ratio greater than or equal to the screening cut point) from a putative negative sample. The sample was confirmed as positive if the percent signal inhibition was greater than or equal to the confirmatory cut point, and the ADA response was said to be specific for marstacimab if the percent signal inhibition was greater than or equal to the confirmatory cut point as a result of exposure to an excess of free drug (i.e., marstacimab). A minimum of 4 independent runs per serum sample were analyzed, with at least 40 lots from individuals deficient in FVIII and FIX. The percent signal inhibition for each sample was calculated using the formula: $(1 - [\text{inhibited sample} \div \text{uninhibited sample}]) \times 100$ (15).

The screening cut point value was determined statistically based on the 95% upper confidence limit after removal of statistical outliers. The confirmatory cut point was determined statistically based on the 99% upper confidence limit of the percent inhibition values after removal of statistical outliers.

Assay response was evaluated in 40 human plasma samples (diluted 1:34) from individuals deficient in FVIII or FIX. The sensitivity of the screening and confirmatory assays was determined by the interpolated concentration at which the PC produced a response equal to the screening cut point and to the confirmatory cut point, respectively. Sensitivity was calculated as: antilog (mean + t0.05, df × SD), where the mean and SD were calculated from the log-transformed concentration at screening and at confirmatory cut points from each sensitivity curve, t0.0× was the t-distribution critical value, and df was the degrees of freedom (15).

Selectivity was assessed as matrix, hemolytic, and lipemic recovery using the screening assay format. Matrix recovery was analyzed from samples of patients with severe hemophilia and NC that was spiked and unspiked at the LPC and HPC levels. Hemolytic samples contain lysed erythrocytes which may impact assessment due to release of intracellular proteins that can interfere with antibody interactions and spectrophotometric detection. Lipemic samples contain accumulated lipoproteins that may interfere with antibody interactions, spectrophotometric detection, and cause hemolysis of samples. Hemolytic and lipemic recovery from low, medium, and high levels of hemolyzed or lipemic matrix were analyzed for unspiked and spiked samples at the HPC and LPC levels. Blank hemolytic samples were spiked with whole blood during the assessment of hemolytic recovery.

Specificity was assessed through drug tolerance and target interference testing. Drug tolerance was generally defined as the amount of marstacimab required to impact the performance of the PC and NC (15). In the validation of Method 3, the drug tolerance limit was defined as the highest concentration of marstacimab that did not suppress responses of the PC samples below the screening cut point. Samples of NC (blank), LPC, HPC, and PC (100 ng/mL and 250 ng/mL) were prepared for analysis by spiking them with marstacimab (0, 25, 50, 100, 200, 300, and 400 μg/mL) in NC, incubating for at least 1 h at room temperature, and freezing for at least 12 h. Target interference limit was defined as the highest concentration of TFPI that does not increase the response of the NC above the screening cut point or decrease the responses of the PC sample below the screening cut point. Samples of the NC, LPC, HPC, and PC (100 ng/mL and 250 ng/mL) were prepared for analysis by spiking with TFPI (0, 125, 250, 1000, 1500, and 2000 ng/mL) in NC, incubating for at least 1 h at room temperature, then freezing for at least 12 h.

Validation of Method 3 also included an evaluation of whether the assay could detect antibodies in a range of sample concentrations without exhibiting a prozone effect. The PC (100 μg/mL in 100% human matrix) was diluted in NC threefold at least eight times, and the serial dilutions were then tested using the screening assay.

Stability of the anti-marstacimab PC was assessed after at least 18 h at room temperature and after at least 7 cycles of freezing (at −20°C and −70°C) for at least 24 h in the first cycle, and at least 12 h in subsequent cycles. Samples were analyzed after the completion of the seventh cycle. Stability of the labeled reagents was assessed after at least 3 cycles of freezing at −70°C for at least 24 h in the first cycle and

at least 12 h in other cycles. The reagents were then used in the assay after the final thaw cycle.

Robustness was assessed by analyzing whether the performance of the PCs and NCs was affected by differences between analysts, reagent lots, plate lots, incubation times, and instrumentation during the course of validation. The goal of evaluating robustness is to determine whether the assay has consistent performance under relevant, real-life standard laboratory conditions (15).

Results

Assay Validation

Validation data for Method 1 sample testing are summarized in Table I. Intra-run, intra-day, and inter-run precision measurements met the acceptance criteria, as all %CVs were less than 10%. The statistical screening assay cut point factor was determined to be 1.08, based on validation data ($n = 50$ normal samples, 5% false positive rate), and the titration cut point factor was 1.11. Following reassessment using predose samples from the phase 1 clinical trial, the average calculated plate cut point factor for sample analysis was 1.19.

While the results of drug tolerance testing indicated that the assay could detect ADAs in the presence of up to 100 μg/mL of circulating drug, the results of target interference testing suggested that the circulating form of TFPI *in vivo* may interfere with the assay, as 150 ng/mL or greater TFPI may generate false positive ADA results. In the first-in-human study (ClinicalTrials.gov identifier: NCT02531815), the highest total TFPI level measured was approaching 350 ng/mL at the highest dose of 440 mg IV (11).

To address this, Method 2, an ECL method utilizing affinity capture elution (ACE) and TFPI blocking, was developed (16, 17). The statistically determined cut point factor for Method 2 was 1.24 for screening and titration. The confirmatory cut point was calculated to be 70.4% inhibition. Sensitivity of the screening assay was 182 ng/mL. However, a LPC at 400 ng/mL was prepared for use in drug and target tolerance experiments, since this was a level that consistently confirmed positive. Validation parameters were within acceptable limits and were generally similar to parameters observed for Method 1 (Table I). Using Method 2, both drug tolerance levels (200 μg/mL *vs.* 100 μg/mL) were increased relative to Method 1. Target tolerance was also improved in that target levels of 50 ng/mL or more yielded false positive results in Method 1, whereas in Method 2 no target interference was seen up to 1200 ng/mL. In the phase 1b/2 study (ClinicalTrials.gov identifier: NCT02974855), the steady-state trough concentrations of marstacimab were between 20.6 and 58.9 μg/mL and the median total TFPI values

measured were < 600 ng/mL across all dose levels over the study duration (12).

The unusually high confirmatory cut point in Method 2 (70.4%) resulted in many screen positive samples not being confirmed positive (i.e., false positives), and can be attributed to a significant level of nonspecific binding that is reduced when excess drug is added. Detection of anti-marstacimab ADAs in human plasma samples evolved further into Method 3, which was an improved ECL-based method that incorporated the use of ChromPure human IgG, as a second specific blocking reagent. ChromPure is a commercial reagent that is purified from normal human serum and was used as an additional specific blocking reagent during ECL.

Validation data for Method 3 are summarized in Table I. The screening cut point factor in Method 3 was 1.26, which was determined after outliers were excluded and the pooled $\log_{10}$ (S/N) data were found to have a non-normal distribution. The confirmatory cut point was 25.6%, which was determined from the data points that remained after exclusion of outliers revealed a normal distribution of pooled percent signal inhibition data. The LPC at 110 ng/mL was consistently positive in both screening and confirmatory assays. Data for other validation parameters in Method 3 were within acceptable criteria and showed improvement compared with validation data from Methods 1 and 2 (Table I). For example, Method 3 had higher values than Method 2 for level of drug tolerance (400 μg/mL *vs* 200 μg/mL). Target tolerance was demonstrated up to 2000 ng/mL in Method 3. Stability of the PC at room temperature for 24 h and through 7 freeze/thaw cycles were within the acceptance criteria (S/N within the 99% confidence interval ranges set during validation). The calculated recovery showed that 10 of 10 samples (blank and spiked) demonstrated matrix recovery; 6 of 6 hemolyzed samples and 6 of 6 lipemic samples also met acceptance criteria specified by the validation plan (HPC [S/N] > LPC [S/N] ≥ SCP). The assay sensitivity, which was the concentration of PC that resulted in a signal equal to the cut point with 95% confidence, was 110 ng/mL No pro-zone effect of the PC was observed over the concentration range that was tested (5.07 ng/mL – 100 mg/mL). Method 3 met acceptance criteria for robustness, with relatively little variation across different sample runs, despite differences in laboratory conditions.

Discussion

Based on the relatively low perceived risk from an ADA response with this drug, the standard recommended approach to multitiered ADA testing was used during the clinical studies (1, 2, 18). As previously reported (11) in a phase 1 study of healthy volunteers who received a single dose of

marstacimab SC or intravenously, the validated Method 1 assay for marstacimab ADA showed 47% of dosed subjects had positive results on days 14 to 42. As the process of ADA assay validation revealed the potential for interference by circulating TFPI ($\geq$ 150 ng/mL to generate false positive ADA results), and study results demonstrated a treatment-mediated elevation of total TFPI (11) it was anticipated that this interference, compounded with other factors, may have contributed to the high frequency of positive results reported in the ADA assay (Method 1). To address target interference, the Method 2 protocol that incorporated ACE procedures was developed and validated to support ongoing phase 2 and 3 clinical studies. Use of the modified methods resulted in a lower incidence of ADA in the phase 1b/2 (11.5%) and phase 2 (0%) studies (12, 19), whereas ADA data was not available for the ongoing phase 3 study.

The MSD platform-based bridging ECL assay with ACE used in Method 2 was further modified during the development of Method 3 to include ChromPure human IgG as an additional specific blocking reagent for blocking or reducing nonspecific background signals. Drug tolerance and target interference were improved in Method 3 compared with Method 2, suggesting that the use of ChromPure reagent improved assay performance. In Method 3, the drug tolerance was fourfold higher than in Method 1 and twofold higher than in Method 2.

Almost all protein-based therapeutics may be immunogenic and may potentially induce the production of ADAs (20). The possible association between ADAs and pharmacokinetics, loss of efficacy and safety has led regulatory bodies to request immunogenicity assessments during the approval process for biotherapeutic agents (20), and recommendations for the validation of ADA detection antibodies have been published (15). In all three methods, matrices that were representative of samples from the target population (i.e., patients with hemophilia or deficient in FVIII and FIX) were used, as recommended in regulatory guidelines (14, 21). Another strength of Methods 1, 2 and 3 was the use of bridging immunoassays, an increasingly common format for ADA detection that overcomes the limitations of sandwich immunoassays (22). In Methods 2 and 3, a bridging immunoassay was used in an ECL platform, allowing detection of anti-marstacimab antibodies when the biotin-labeled marstacimab was used as a capture reagent on a streptavidin-coated plate and ruthenium-labeled marstacimab was used as the detector reagent (Fig. 2) (22). The ADAs in the sample were detected because they formed a bridge between the biotin-labeled marstacimab and the ruthenium-labeled marstacimab. Additional advantages of bridging immunoassays are the ability to detect the majority of antibody isotypes in the same assay and the convenience of using a cross-species control, if needed, without requiring separate species-specific detection reagents (22). This was exemplified in Methods 2 and 3, which used mouse anti-TFPI antibody clone 106–104 as the PC for the bridging immunoassay.

Limitations

A human ADA PC was not available. Instead, validation was conducted using surrogate PC antibodies (a rabbit anti-TFPI antibody as the PC for Method 1 and a mouse anti-TFPI antibody as the PC for Methods 2 and 3), which may not fully reflect the *in vivo* formation of ADAs in patients with hemophilia in terms of antibody class, subclass, affinity, avidity, and magnitude, and in the context of concomitant medications and other patient characteristics (14). Secondly, response signals from pre-existing marstacimab ADAs and potential nonspecific interference may still complicate the detection of marstacimab ADAs that are induced after starting treatment with marstacimab. Therefore, comparison of predose and postdose samples responses will be important in identifying meaningful treatment-related induction of marstacimab ADAs.

Conclusion

The development of robust, sensitive, specific, and selective assays to measure ADA responses is a key part of the clinical development of marstacimab. A quasi-quantitative immunogenicity ECL-based assay to detect the presence of anti-marstacimab ADAs in sodium citrate human plasma samples was developed in a clinical-stage appropriate manner. It is anticipated that this ADA detection assay will be a useful adjunct in evaluating the efficacy, safety, and pharmacokinetics of marstacimab in patients with hemophilia and will be important in understanding the impact of ADA induction (if any) on these parameters.

Author Contribution Conducted experiments: JAD, YX
Performed data analysis: JAD, DJ, YX
Participated in data interpretation: JAD, BMG, DJ
Contributed to the writing of the manuscript: JAD, BMG
All authors collaborated in the preparation of the manuscript, and critically reviewed and provided revisions to the manuscript. All authors had access to the data and assume responsibility for the completeness and accuracy of the data and data analyses. All authors granted final approval of the manuscript for submission.

Declarations

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